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The Function of Bile Salts in Fat Absorption

THE SOLVENT PROPERTIES OF DILUTE MICELLAR SOLUTIONS OF CONJUGATED BILE SALTS

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That bile salts influence the absorption of dietary fat has been known for many years, but the mechanism of their action has remained unclear. Hartley (1936) proposed the term 'amphipathic' to refer to compounds possessing dissymmetric polar and non-polar regions, which, because of their unusual molecular structure, exhibit a certain characteristic behaviour in water under appropriate conditions. Such substances behave as detergents when micellar solutions are formed above their respective Krafft points. Hartley (1936) pointed out that bile salts should be classified as amphipathic compounds. Subsequently, dye-solubility studies by McBain, Merrill & Vinograd (1941) as well as osmotic-pressure measurements (Roepke & Mason, 1940) confirmed Hartley's prediction that micellar aggregation should take place in aqueous bile salt solution above the critical micellar concentration. For reviews on micelle formation, see Hartley (1936, 1955), McBain & Hutchinson (1955), Pankhurst (1953) and Garrett (1961).

Ekwall, Sten & Norman (1956) and Ekwall, Fontell & Sten (1957) have studied the detergent properties of certain of the bile salts, and have compared them with paraffin-chain soaps. The development of greatly improved synthetic methods (Norman, 1955) and chromatographic methods (Norman, 1953; Sjövall, 1959*a*) enabled Norman (1960) to compare the critical micellar concentrations of the glycine- and taurine-conjugated bile acids, which are predominant in human bile.

Micellar solubilization of the products of pancreatic lipolysis has been proposed as an important step in fat absorption (Bergström & Borgström, 1955), and recent work has demonstrated a micellar phase of bile salt, fatty acid, monoglyceride and cholesterol in human-intestinal content during fat absorption (Hofmann & Borgström, 1962).

The present paper gives observations on the solvent properties of dilute micellar bile salt solutions *in vitro* under conditions similar to those present in the human small-intestinal lumen during fat absorption, with respect to Na^+ ions (0·15M), pH (6·3) and temperature (37°) (Borgström, Dahlqvist, Lundh & Sjövall, 1957). With these experimental conditions, micellar solutions are not formed by the salts of the unconjugated bile acids (Hofmann, 1961*a*, *b*) and the experiments described below deal with only the glycine and taurine conjugates, the forms occurring in man (Sjövall, 1959*b*).

The predominant products of pancreatic lipolysis are amphiphiles, that is, compounds also having dissymmetric polar and non-polar regions, but which are poorly soluble in water (Lawrence, 1959, 1961). They are soluble in certain detergent solutions forming a liquid-crystal phase or coacervate which contains the compound, detergent and water when the amphiphile is present in excess. Because of the complexity of amphiphile–detergent interactions, it seemed important to characterize the solvent properties of micellar bile salt solutions with respect to the simplest solutes possible. A representative non-polar solute, *trans*-azobenzene (Hartley, 1938*b*), and a polar or amphiphilic compound, glycerol 1-mono-oleate (Hofmann, 1961*a*), were chosen as prototypes. Alkyl alcohols, the amphiphilic substances most commonly employed

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in similar studies (McBain & Hutchinson, 1955), could not be used, as they do not exhibit amphiphilic behaviour in dilute bile salt solutions.

Measurements have been made of the solubility of these two solutes in dilute solutions of the six bile salts occurring in man as well as a mixture of them simulating human-intestinal content. The critical micellar concentrations have been determined. Observations have also been made on the influence of Na^+ ions, temperature, pH, Ca^{2+} ions and glucose.

The solvent properties of bile salt solutions have been compared in some respects with those of three typical anionic detergents.

A preliminary report of some of these findings has been published (Hofmann, 1961*a*).

MATERIALS AND METHODS

Abbreviations

trans-Azobenzene is termed azobenzene. Glycerol 1-mono-oleate is also termed mono-olein.

Assay of purity

Thin-layer chromatography (Stahl, 1961; Mangold, 1961; Hofmann, 1962*a*, *b*, 1963*b*) was used extensively for assaying the purity of compounds. R_F values are given to one figure only because of their wide variability with this chromatographic technique.

Solutes

Azobenzene. Azobenzene (Merck) contained a few per cent of the *cis*-isomer when examined by thin-layer chromatography with heptane–benzene (1:1, v/v). R_F values: *trans*-azobenzene, 0·8; *cis*-azobenzene, 0·3. Purification was by the method of Hartley (1938*a*). The final crystals, m.p. 67°, were pure *trans*-azobenzene; no detectable impurities could be seen when 200 μg. was chromatographed.

Glycerol 1-mono-oleate. Glycerol 1-mono-oleate (Distillation Products, Rochester, N.Y., U.S.A.) contained about 1% of diglyceride (both 1,2- and 1,3-isomers) and 1% of fatty acid (by thin-layer chromatography and titration with alkali). By periodic acid oxidation (Pohle & Mehlenbacher, 1952; Desnuelle & Constantin, 1952) and perchloric acid-induced isomerization (Hartman, 1960), it contained about 3% of the 2-isomer. Its fatty acid composition was: octadecaenoic acid, 76%; hexadecaenoic acid, 12%; octadecadienoic acid, 3%; hexadecanoic acid, 3%; tetradecanoic acid, 3%; tetradecaenoic acid, 2% (by gas–liquid chromatography). This preparation was used despite its heterogeneity, as it was available in large quantities so that the same amphiphile could be used in all experiments. A much purer sample of glycerol 1-mono-oleate was obtained from Dr D. Beck (Procter and Gamble Co., Cincinnati, Ohio, U.S.A.) which was 99% pure with respect to both class and homologue composition. The solubility of this pure mono-olein in bile salt solutions was identical with that of the impure mono-olein.

Conjugated bile salts. Methods for the preparation of pure conjugated bile acids are still being improved (Hofmann, 1963*a*). Sodium glycodeoxycholate, sodium taurodeoxycholate and sodium glycocholate had been crystallized; sodium taurocholate, sodium glycochenodeoxycholate and sodium taurochenodeoxycholate were freeze-dried preparations. The final white powder dissolved without residue in water, chloroform–methanol (1:1, v/v) or hot ethanol to give an absolutely clear solution without opalescence. The chief impurity in the preparations used was free bile acid, usually less than 2%. Experiments performed with the deliberate addition of free bile acid have shown that this small amount of contaminating free acid should not influence results.

The assessment of purity was by thin-layer chromatography (Hofmann, 1962*a*). The solvent mixture for separating free bile acids is called 'system 1'; that for conjugated bile acids, 'system 2'. The free bile acids were purified before conjugation, as pure conjugated bile acids cannot be prepared from impure free acids.

Preparation of free bile acids

Cholic acid. Cholic acid (Riedel de Haen, Hannover, Germany) was 93–96% pure by thin-layer chromatography, the impurities being deoxycholic acid, chenodeoxycholic acid and an unidentified, slightly less polar, impurity with R_F 0·2 in system 1. The acid was purified by crystallization (Hofmann, 1963*a*).

Deoxycholic acid. Deoxycholic acid (T. Schuchardt, Munich, Germany) was 97–98% pure by thin-layer chromatography, its chief impurity being cholic acid. Commercial samples from other sources were less pure. Most of the deoxycholic acid used was purified by preparing 3α,12α-diacetoxydeoxycholic acid 5β-methyl ester (Reichstein & Sorkin, 1942), followed by saponification and crystallization of the resultant free acid from acetone. Subsequently, it was observed that a single crystallization of the commercial product from 80% (v/v) acetone gave a product that was at least 99% pure.

Chenodeoxycholic acid. Chenodeoxycholic acid was prepared from cholic acid by Wolff–Kishner reduction of 3α,7α-dihydroxy-12-oxo-5β-cholanic acid, followed by adsorption chromatography of the methylated impure acid on alumina. The final product, m.p. 146° from ethyl acetate or 119° from ethyl acetate–heptane, had a purity greater than 99% (Hofmann, 1963*a*).

Preparations of conjugates

Conjugation with glycine and taurine was performed as described by Norman (1955) with modifications (Hofmann, 1963*a*).

Taurine conjugates. The reaction mixture, after evaporation, was dissolved in a large volume of chloroform–methanol (2:1, v/v), ethanol being added if necessary to give one phase, and filtered after about 15 min. After evaporation without warming, the reaction mixture was dissolved in 90% (v/v) ethanol and percolated slowly through a Dowex 50-W column (H^+ form, prepared in 90% ethanol) to remove the tri-*n*-butylamine. The effluent was carefully brought to pH 4–5 with sodium ethoxide, refiltered after 30 min. if a precipitate of sodium chloride had formed, evaporated without warming and dissolved in water. If turbid, the solution was extracted twice with ethyl acetate, then twice with ether. It was then percolated through a second Dowex 50-W column (H^+ form, prepared in water), extracted rapidly with

ethyl acetate (Anderson, 1962) and twice with ether, and brought to pH 5 with sodium hydroxide. After removal of the remaining ether by a rotary evaporator, the solution was freeze-dried, then dried at room temperature *in vacuo* over phosphorus pentoxide and sulphuric acid. The freeze-dried products were white free-flowing powders and were not hygroscopic.

Glycine conjugates. The reaction mixture was neutralized and evaporated without warming to near dryness. It was then dissolved in 50% (v/v) ethanol and the pH adjusted to 10–11 with sodium hydroxide. After three extractions with ether–light petroleum (b.p. 60–70°) (1:1, v/v), the aqueous phase was adjusted to pH 7 and most of the alcohol removed by a rotary evaporator. The acid was then precipitated from solution by the addition of hydrochloric acid to give pH 3–4.

Glycocholic acid and glycodeoxycholic acid, although impure, could invariably be crystallized at this point by trituration with acetone. The crystals were removed by filtration and washed well with water.

All preparations of glycine conjugates were usually contaminated with free acids (usually 10–15%), which could be removed by liquid–liquid extraction of an aqueous solution adjusted in pH so that all free bile acid present was un-ionized and the majority of glycine conjugate was ionized. Extraction of an unbuffered or acidic aqueous phase proved unsuccessful because of the similar partition coefficients of the free bile acids and their respective glycine conjugates.

Glycocholic acid was suspended in water and neutralized to pH 7 with sodium hydroxide. The pH was then adjusted to 4–5 by the dropwise addition of 1 N-hydrochloric acid with continuous agitation. The clear solution was extracted repeatedly with small volumes of ethyl acetate until thin-layer chromatography of the lower phase showed negligible cholic acid.

The solution was then acidified to give pH 2, and the glycocholic acid crystallized as described above. The crystals were washed thoroughly with water and recrystallized from aqueous acetone.

Glycodeoxycholic acid was purified similarly, except that the pH was adjusted to 5–6 and the extraction was with ether.

The final product is still impure, being contaminated by a more polar impurity, which is probably a glycylglycine conjugate. This cannot apparently be removed by crystallization or solvent extraction, but can be removed by chromatography (Hofmann, 1963*a*). The samples of glycocholic acid and glycodeoxycholic acid used were not chromatographed but had been recrystallized several times; they contained about 1–2% of this impurity.

The preparation of glycochenodeoxycholic acid and sodium glycochenodeoxycholate was as described by Hofmann (1963*a*). The glycochenodeoxycholate used in these experiments was purified by a liquid–liquid-extraction scheme similar to that described above. The final product was 95–96% pure, containing a few per cent of the free acid and a few per cent of the unidentified more-polar impurity.

All conjugated bile acids were dried for 24 hr. at 100° *in vacuo* over phosphorus pentoxide.

Sodium oleyl taurate. This was synthesized by Dr L. Krabisch by using the method outlined for preparation of the taurine-conjugated bile acids. Oleic acid, pure by gas–liquid chromatography, was used. The final product was homogeneous by thin-layer chromatography with system 2, R_F 0·3. Its m.p. after drying was 114–115° (decomp.).

Sodium n-*octylbenzene*-p-*sulphonate.* This was given by Dr M. E. Ginn (Monsanto Chemical Co., Dayton, Ohio, U.S.A.). It contained no alkyl alcohol when examined by thin-layer chromatography. With system 2, it moved as a single spot, R_F 0·4.

Sodium lauryl sulphate. Sodium lauryl sulphate ('technical pure') was a gift of Du Pont, Wilmington, Del., U.S.A. It moved as a single spot with system 2, R_F 0·4. Its 'class' composition was stated by the manufacturer to be: lauryl sulphate, 96%, sodium chloride, 0·05% and lauryl alcohol, 0·15%; and its homologue composition: duodecanoic acid, 65%; tetradecanoic acid, 28%; hexadecanoic acid, 7%.

Solutions

Aqueous solutions of conjugated bile salts were prepared by dissolving 2 m-moles of bile salt or bile salt derivative and 5·5 m-moles of sodium chloride in 50 ml. of distilled water. The final water-clear solutions were thus 0·04 M with respect to bile salt and 0·15 M with respect to Na^+ ion; they were kept refrigerated.

A solution of bile acids simulating human-intestinal content was prepared, based on the analyses of Sjövall (1959*b*), by mixing the 0·04 M-bile salt solutions described above. It contained, by vol.: sodium glycocholate, 30%; sodium glycochenodeoxycholate, 30%; sodium glycodeoxycholate, 15%; sodium taurocholate, 10%; sodium taurochenodeoxycholate, 10%; sodium taurodeoxycholate, 5%. The final solution was therefore 0·04 M with respect to bile salt concentration and 0·15 M with respect to Na^+ ion.

Aqueous solutions of sodium *n*-octylbenzene-*p*-sulphonate and sodium lauryl sulphate were prepared similarly to a concentration of 0·04 M with respect to detergent and 0·15 M with respect to Na^+ ion. They were kept at 37° after preparation as the Krafft points of these solutions are above room temperature.

Aqueous solutions of sodium oleyl taurate were prepared with 1 m-mole of detergent and 6·5 m-moles of sodium chloride in 50 ml. of distilled water, as more concentrated solutions were extremely viscous. The final concentration was 0·02 M with respect to detergent and 0·15 M with respect to Na^+ ion; they were kept at room temperature.

Buffer solutions

Buffer solutions were prepared by mixing the following solutions in appropriate volumes to give the desired pH; the pH was recorded with a Radiometer (Copenhagen, Denmark) pH-meter. For pH 3–5·8: solution 1, 0·3 M-citric acid in 0·3 M-NaCl; solution 2, 0·1 M-sodium citrate. For pH 5·8–8·0: solution 1, 0·3 M-NaH_2PO_4; solution 2, 0·15 M-Na_2HPO_4. For pH 6·0–7·0 in experiments in which Ca^{2+} ions were present: solution 1, 0·3 M-sodium hydrogen maleate; solution 2, 0·3 N-NaOH. For pH 7·8–9·0: solution 1, 0·15 M-boric acid in 0·3 M-NaCl; solution 2, 0·075 M-sodium borate in 0·15 M-NaCl. The solutions prepared were thus 0·3 M with respect to Na^+ ion. They were diluted 1:1 (v/v) with distilled water immediately before use.

Solubility measurements

Experiments were performed in 10 ml. glass ampoules (Johnsen and Jörgensen Ltd., London) which were sterilized by washing with alcohol and by drying at 110°.

They were of sufficient uniformity in diameter and thickness to be usable as cuvettes in a spectrophotometer.

Solute (see below) followed by the bile salt or detergent solution and then buffer were added with a syringe to give a final volume of 5·0 ml., and the ampoules sealed. They could be kept for a month at 37° without evidence of bacterial growth.

Sodium phosphate buffer, pH 6·3 (0·15 M with respect to Na^+ ion), prepared as described above, was used for all experiments with bile salts or detergents, where pH was not a variable or Ca^{2+} ions were not present.

Measurement of azobenzene solubility

Because the *trans*-isomer spontaneously isomerizes to the *cis*-form (Hartley, 1938*a*) and because the latter has a far greater extinction at 440 mμ, exposure to light was minimized. The ampoules were prepared with appropriate detergent and buffer and a few crystals of azobenzene were added in a dark-room. Each ampoule was then wrapped in aluminium foil, sealed, placed in closed boxes and shaken. The boxes rested at a 45° angle on the shaker so that neither solute nor solvent became occluded in the tips of the ampoules. Equilibrium was reached in less than 2 days at 37°, and the extinction values were stable for several weeks. Equilibration and all subsequent procedures such as spectrophotometry or filtration were performed in a 37° dark-room with a yellow lamp for illumination.

The dissolved azobenzene was determined spectrophotometrically at 440 mμ by using a Coleman Junior spectrophotometer equilibrated at 37°. Standards containing 0·08–0·64 μmole of azobenzene/ml. of dioxan were read daily for some months and showed no change in extinction. Systems were read against distilled water and suitable blanks were included. If the ampoule was allowed to stand undisturbed for 30 min. the solution became free of suspended particles. The ampoule could be used directly for extinction determinations. Identical results were obtained whether the ampoules were read directly or the contents first filtered through Munktell's 20H filter paper and read in cuvettes. If there was any doubt about the solute's behaviour, the ampoule contents were filtered. This method does not work as well when detergent is not present or is at a concentration below its critical micellar concentration, but neither does filtration, as supersaturated solutions occur easily. The solubility of azobenzene in buffer alone always showed a variation of 10%; this was of little significance to the interpretation of the experiment. Almost all experiments were performed in duplicate. When detergent was present, agreement between duplicates was excellent, usually within 2–3%.

The validity of this method was checked as follows. A portion (1·6 μmoles) of azobenzene was added to a series of ampoules from a dioxan stock solution, and the dioxan removed by evaporation. Sodium glycodeoxycholate and phosphate buffer were added such that the final solutions were 16, 24 and 32 mM with respect to bile salt concentration. After shaking for 1 day, the azobenzene was completely dissolved in all ampoules, the contents of which showed identical extinctions at 440 mμ. These results indicated that sodium glycodeoxycholate at these concentrations had no effect on the azobenzene extinction at 440 mμ.

A standard calibration curve for azobenzene, with a fixed bile salt concentration of 24 mM-sodium glycodeoxycholate, was prepared by this method. A straight line passing through the origin was obtained with a slope of about 8% less than a similar calibration curve obtained by preparing a series of ampoules in which the dioxan stock solution was merely diluted to 5·0 ml. with dioxan.

Analogous experiments showed that the following had no effect on the extinction of azobenzene in dilute micellar solution: the typical anionic detergents, glucose, the buffer solutions and Na^+ ions, up to a concentration of 400 mM (cf. Sjöblom, 1956).

Measurement of mono-olein solubility

Mono-olein solubility was determined turbidimetrically; equilibrium in detergent solutions occurred within 1 hr. with shaking. Mono-olein is insoluble in buffer alone, although, like lecithin, it forms hydrates giving myelin figures or bimolecular leaflets (Lawrence, 1959, 1961; Hofmann, 1961*a*, *b*; Stoeckenius, Schulman & Prince, 1960). In buffer alone, or in most detergent solutions below their critical micellar concentrations, mono-olein remains adherent to the walls of the glass vessel. In bile salt solutions or those of certain detergents, it dissolves to a considerable extent forming water-clear micellar solutions. When an excess is present a turbid solution results, the extinction of which at 550 mμ rises linearly in proportion to the amount of excess present. This turbid state represents a coacervate in equilibrium with a micellar solution.

Solubility was determined by preparing a series of ampoules containing increasing amounts of mono-olein for each detergent concentration. The extinction at 550 mμ of those ampoules containing an excess was plotted and extrapolated to its intersection with the line extrapolated from the values obtained where the mono-olein was completely dissolved. The extinction of the latter was essentially zero. The turbidimetric method described (cf. Klevens, 1950; Ekwall & Vittasmäki, 1956) is empirical but seems to be satisfactory. With bile salt solutions and mono-olein as the solute, the extinction rises linearly only for small amounts in excess. With sodium *n*-octylbenzene-*p*-sulphonate and sodium lauryl sulphate, the increase in extinction caused by excess was not linear and solubility values could be estimated only roughly. The method cannot be used below the critical micellar concentration of the system. Most experiments were done in duplicate with an agreement of ±2% between duplicates or ±5% between replicates.

Influence of pH

An appropriate amount of bile salt solution was added to the solute, then buffer to give a final volume of 5·0 ml. The final concentration of bile salt was well above the critical micellar concentration in each instance. In the experiments with mono-olein, a series of ampoules containing increasing amounts of mono-olein was used for each pH. Control experiments showed that the solubility of azobenzene in these buffer solutions alone was constant from pH 3·0 to 9·0. Mono-olein is insoluble in buffer alone.

Influence of Na^+ *ion concentration*

In the first type of experiment, the bile salt concentration was held constant while the counter-ion concentration was varied. 0·04 M-Bile salt solutions were prepared in distilled water. To ampoules containing azobenzene were added

samples of 0·75 M-NaCl, a constant volume of this bile salt solution, sodium phosphate buffer (pH 3·6; 0·15 M with respect to Na^+ ion) and water to give a total volume of 5·0 ml. The final bile salt concentration was well above the critical micellar concentration, being 8 mM for dihydroxy bile salts, 10 mM for 'simulated intestinal content' and 12 mM for trihydroxyl bile salts. The total Na^+ ion concentration, referred to as counter-ion concentration, was calculated retrospectively. For experiments with mono-olein as the solute, a series of ampoules containing increasing amounts of mono-olein was prepared for each counter-ion concentration.

In the second type of experiment, performed only with azobenzene in sodium glycocholate in order to measure the influence of Na^+ ion concentration on the critical micellar concentration as well as the saturation ratio, a range of bile salt concentrations was prepared for several fixed total counter-ion concentrations. Three different counter-ion concentrations were used (0·11, 0·185 and 0·25 M). To each ampoule was added x ml. of 0·04 M-bile salt solution, which had been prepared in distilled water, $(2·5 - x)$ ml. of 0·04 M-NaCl, samples of 0·75 M-NaCl, 0·5 ml. of phosphate buffer (pH 6·3; 0·15 M with respect to Na^+ ion) and water to give a volume of 5·0 ml.

The effect of increasing the Na^+ ion concentration on the solubility of azobenzene in buffer alone was measured, but the fall in azobenzene solubility noted was negligible compared with the effect of Na^+ ion concentration on micellar azobenzene solubility with the bile salt concentration employed. No effect of Na^+ ion concentration on azobenzene extinction was detected (but see Hartley, 1938*b*).

Influence of temperature

Measurements of the solubility of azobenzene were made at 23° and 37°. Generally an experiment was equilibrated at 37°, read in the spectrophotometer, then equilibrated at 23°. Results were identical if the experiment was equilibrated at 23° first.

Influence of Ca^{2+} ions

A solution 0·10 M with respect to Ca^{2+} ions and 0·05 M with respect to Na^+ ions was prepared; thus its total counter-ion concentration was 0·15 M. Increasing amounts of this solution were added to a series of ampoules containing a single detergent concentration, sodium maleate buffer and azobenzene. The final Ca^{2+} ion concentrations ranged from 2 to 10 m-equiv./l. Similar experiments with mono-olein were performed.

Influence of glucose

Samples of a glucose solution (16·66 g./100 ml. of 0·15 M-NaCl) were added to ampoules containing a fixed bile salt concentration, phosphate buffer and azobenzene. The final glucose concentrations ranged from 33 to 100 mg./ml., and thus were in the same range as those in human-small-intestinal content during digestion and absorption (Borgström *et al.* 1957).

RESULTS

Azobenzene solubility in bile salt solutions. The solubility of azobenzene in the individual bile salt solutions and 'simulated intestinal content' is shown in Fig. 1. Such curves, in which the solubility of an oil-soluble compound is plotted against detergent concentration, are termed solubilization curves and they show a characteristic form. The flat first portion of the curve represents the solubility of the solute in water or buffer alone. The second portion where the solubility of the substance increases markedly for a slight increase in detergent concentration corresponds to the first occurrence of micelle formation. The third and predominant portion shows a linear or approximately linear increase with increasing detergent concentration. The detergent solution at this concentration range is now largely micellar; added detergent is believed to form additional micelles of the same size while the concentration of unassociated detergent anions remains approximately constant (Hartley, 1936; Shinoda & Hutchinson, 1962). The slope of the linear portion of the curve represents the ratio of micellar solute to micellar detergent and is termed

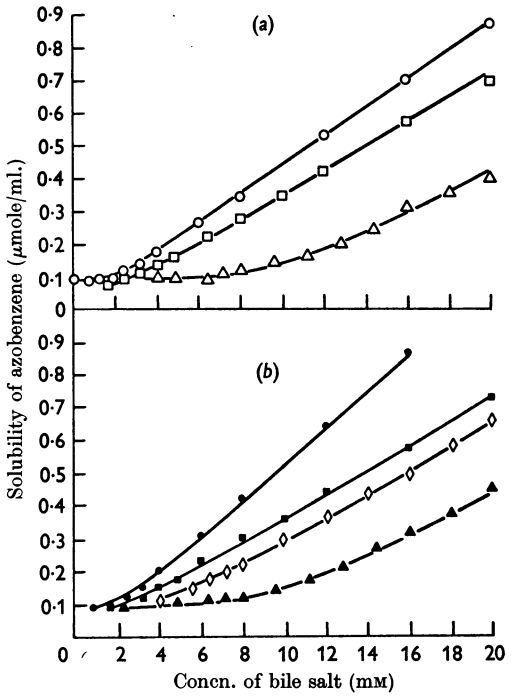

Fig. 1. Effect of bile salt concentration on azobenzene solubility (in phosphate buffer, pH 6·3; concn. of Na^+ ion, 15 M; 37°). (*a*) Taurine conjugates: ○, sodium taurodeoxycholate; □, sodium taurochenodeoxycholate; △, sodium taurocholate. (*b*) Glycine conjugates: ●, sodium glycodeoxycholate; ■, sodium glycochenodeoxycholate; ▲, sodium glycocholate; ◇, 'simulated intestinal content'. The inflexion of a curve denotes the onset of micellar solubilization and indicates the critical micellar concentration; its slope, the saturation ratio (moles of micellar azobenzene/mole of micellar bile salt).

the saturation ratio (cf. Hartley, 1938*b*). The saturation ratios for the different bile salts and 'simulated intestinal content' with azobenzene as the solute are listed in Table 1.

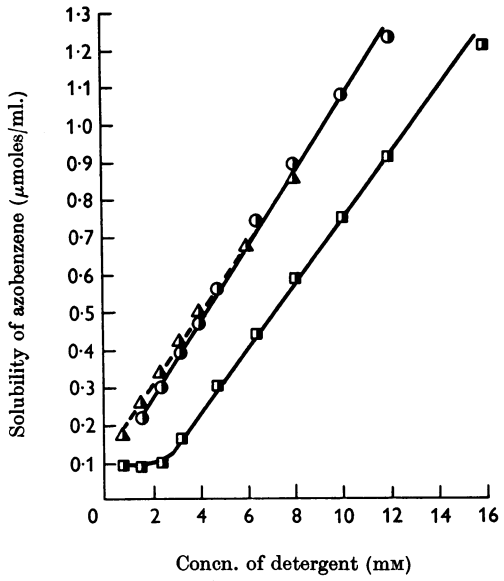

Fig. 2. Solubilization curves of azobenzene in three typical anionic detergents (in phosphate buffer, pH 6·3; concn. of Na^+ ion, 0·15M; 37°). △, Sodium oleyl taurate; ◑, sodium lauryl sulphate; ◨, sodium *n*-octylbenzene-*p*-sulphonate.

By convention, the critical micellar concentration is usually determined by extrapolating the linear part of the solubilization curve to its intersection with the extrapolated buffer or water solubility. This can be done for typical detergents where the solubilization curves show a sharp inflexion over a narrow concentration range and then become linear. Fig. 2 shows the solubility of azobenzene in three typical anionic detergents under the same experimental conditions as those described in Fig. 1. The critical micellar concentrations were determined and are shown with the saturation ratios in Table 1.

For bile salt solutions, however, the increase in azobenzene solubility is gradual as micelle formation begins. Further, the solubilization curves of the trihydroxy conjugates for azobenzene are not linear (Fig. 1). The critical micellar concentrations (Table 1) were obtained by extrapolating both the linear and the preceding part of the curve and taking an arithmetic mean. These are useful for comparative purposes, but a critical-micellar-concentration range would be a more meaningful term.

The saturation ratio of 'simulated intestinal content' and the trihydroxy conjugates increases with concentration. The values in Table 1 refer to the slope where the curve is nearly linear at a concentration well above the critical micellar concentration, but it is apparent that these values are somewhat arbitrary.

The influence of the number and position of

Table 1. *Critical micellar concentration and saturation ratio of conjugated bile salts, 'simulated intestinal content' and three typical anionic detergents for* trans-*azobenzene and* 1-*mono-olein*

These values were derived from the results shown in Figs. 1–3. The experimental conditions were: concn. of Na^+ ions, 0·15M; pH 6·3; 37°. The respective critical micellar concentrations are probably approximately correct for the majority of non-polar or amphiphilic solutes with the experimental conditions described. The saturation ratios are useful for comparing the bile salts with each other, but the absolute values apply only to these solutes under these conditions and would be different if other solutes were used. However, the marked difference between non-polar and amphiphilic solutes should still be observed. N.D., Not determined.

	Critical micellar concentration (mM)		Saturation ratio (moles of micellar solute / moles of micellar bile salt or detergent)	
	Azobenzene	Mono-olein	Azobenzene	Mono-olein
Sodium glycodeoxycholate	1·9	0·6	0·056	1·7
Sodium taurodeoxycholate	1·9	0·6	0·044	1·7
Sodium glycochenodeoxycholate	2·4	0·8	0·036	1·7
Sodium taurochenodeoxycholate	2·5	0·8	0·036	1·6
Sodium glycocholate*	8·0	4·2	0·034	1·4
Sodium taurocholate*	10·0	4·2	0·024	1·4
'Simulated intestinal content'*	3·5	1·4	0·035	1·4
Sodium *n*-octylbenzene-*p*-sulphonate	2·3	N.D.	0·085	0·9
Sodium oleyl taurate	0·1	N.D.	0·100	0·3
Sodium lauryl sulphate	0·4	N.D.	0·109	0·4

* As noted in the text, the critical micellar concentrations and saturation ratios for azobenzene of the trihydroxy conjugates and 'simulated intestinal content' cannot be measured accurately because of the gradual inflexion as well as the non-linearity of the solubilization curve.

hydroxyl groups on critical micellar concentration and saturation ratio is evident in Fig. 1 and Table 1. A glycine conjugate has a slightly lower critical micellar concentration and higher saturation ratio than its corresponding taurine conjugate, but the difference is not great. The saturation ratios for azobenzene of the typical anionic detergents are two- to three-fold those of the bile salts.

The critical micellar concentrations given represent the values for the different bile salts with this solute only and under these experimental conditions, but would not differ greatly for any non-polar solute. The saturation ratios for other non-polar solutes might be different, however (McBain & Hutchinson, 1955; Sjöblom, 1956), although the differences between the individual bile salts should show the same trend.

Mono-olein solubility in bile salt solution. Fig. 3 shows the solubility of mono-olein in the individual bile salt solutions and 'simulated intestinal content'.

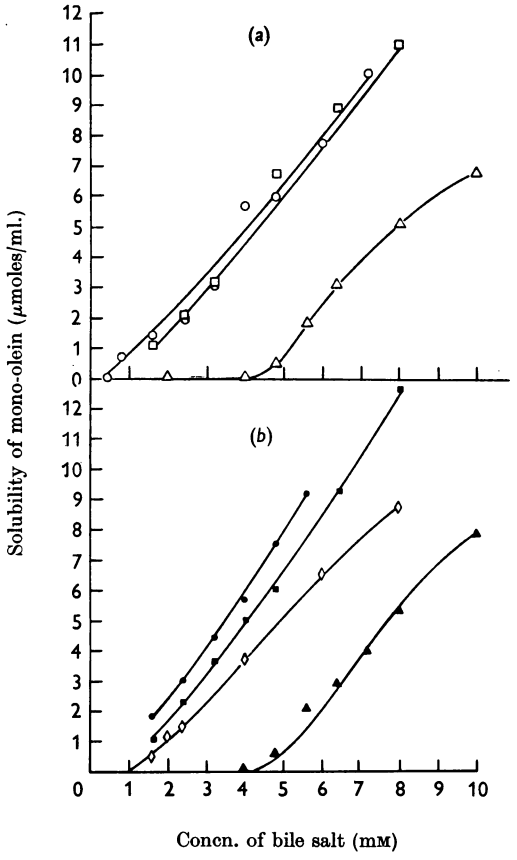

Fig. 3. Effect of bile salt concentration on mono-olein solubility (in phosphate buffer, pH 6·3; concn. of Na^+ ion, 0·15 M; 37°). (*a*) Taurine conjugates; (*b*) glycine conjugates: symbols for experimental points are as in Fig. 1.

The critical micellar concentrations and saturation ratios are given in Table 1. The saturation ratios for mono-olein in bile salt solutions are many times greater than those of azobenzene, indicating the amphiphilic nature of mono-olein. The critical micellar concentration of a ternary system of detergent–amphiphile–water is appreciably lower than that of a detergent–non-polar solute–water system (Lawrence & Stenson, 1957).

As with azobenzene, the critical micellar concentrations of the dihydroxy conjugates are appreciably lower than those of the trihydroxy conjugates, whereas the saturation ratios of the former are slightly higher. At higher bile salt concentrations the glycine conjugates seem to show somewhat higher saturation ratios than their respective taurine conjugates.

The critical micellar concentrations given would not differ greatly for other amphiphilic solutes, e.g. selachyl alcohol, monolinolein or lecithin, under the same experimental conditions. Saturation ratios, however, might be significantly different.

Mono-olein solubility in typical anionic detergent solutions. With micellar solutions of typical anionic detergents only a small amount of mono-olein dissolved into micellar solution. The saturation ratios were considerably lower than those obtained for mono-olein in bile salt solutions (Table 1).

Azobenzene solubility in solutions of bile salt plus mono-olein. The micellar solubility of a non-polar solute in a detergent solution is increased by the addition of amphiphile, this being largely due to the increased hydrocarbon interior of the expanded micelle (Klevens, 1950; McBain & Hutchinson, 1955). Fig. 4 shows the effect of added mono-olein on the solubility of azobenzene in different bile salts, as well as sodium *n*-octylbenzene-*p*-sulphonate. The concentration of monoglyceride at which the system became turbid is indicated, and these values represent the bile salt or sodium *n*-octylbenzene-*p*-sulphonate solution becoming saturated with amphiphile.

There is a marked increase in azobenzene solubility which is directly proportional to the amphiphile added. Similar results were obtained with sodium lauryl sulphate, but, as noted, with this detergent as well as with sodium *n*-octylbenzene-*p*-sulphonate, the amount of mono-olein that dissolved in these solutions without inducing turbidity was considerably smaller. If a saturation ratio is calculated for bile salt solution plus mono-olein with azobenzene as solute, in which the total detergent concentration is taken as the sum of bile salt plus mono-olein, the following saturation ratios are obtained for bile salt micelles saturated or nearly saturated with mono-olein: sodium

taurodeoxycholate, 0·96; sodium taurocholate, 0·59; 'simulated intestinal content', 0·95. Thus it is apparent from Table 1 that the bile salt micelle, when saturated with mono-olein, may have a solvent power for azobenzene comparable with the micelle of the typical anionic detergents studied.

The solubility of mono-olein in the bile acid micelle is uninfluenced by previous saturation of the micelle with azobenzene.

Influence of pH *on azobenzene and mono-olein solubility in bile salt solutions.* The taurine conjugates have pK_a about 2 (Josephson, 1933), and, despite the influence of micellar association on ionization, should be fully ionized above pH 4. The glycine conjugates have pK_a values between 3 and 4 (Josephson, 1933). The lowering of the pK_a of the unconjugated acids from 5–6 to 3–4 as a result of conjugation with glycine may be important physiologically (Hofmann & Borgström, 1962). When a micellar solution of a glycine conjugate is slowly acidified, the undissociated acid formed remains dissolved in the anionic micelles. When the micelle becomes saturated a liquid-crystal state is not formed, but the un-ionized acid precipitates out of solution. This statement is true for glycocholate and glycochenodeoxycholate but probably not for glycodeoxycholate, where the striking increase in viscosity occurring on acidification suggests the formation of helical aggregates (cf. Rich & Blow, 1958). The behaviour of glycine conjugates during acidification is dependent on the solubility of the un-ionized acid in buffer and the micelle, the bile salt concentration, as well as other factors influencing micelle formation (Ekwall, Lindström & Setälä, 1951). With 'simulated intestinal content', the micellar taurine conjugate anions hold un-ionized glycine conjugate molecules in solution during acidification. With saturation of the micelle, precipitation occurs. In 'simulated intestinal content' only one species of micelle is assumed to be present, which is composed of all the anionic types present (Mysels & Otter, 1961). Taurine conjugates are not precipitated from solution by acidification. Their aqueous solutions may be converted completely into the acid form by passage over a cation-exchange column in the H^+ form (Anderson, 1962), and they remain in solution. With azobenzene as solute, glycodeoxycholate (8 mM) was precipitated at pH 5·4 and not at pH 5·8; glycocholate (12 mM) remained in solution at pH 5·0; 'simulated intestinal content' (8 mM) was precipitated at pH 4·2 and not at pH 4·6. The pH had no influence on the solubility of the non-ionized solute azobenzene in bile salt solutions. Mono-olein showed identical solubility in sodium taurodeoxycholate (2·4 mM) from pH 5·5 to pH 7·0. Below pH 4 and above pH 9, spontaneous hydrolysis occurs (Hofmann, 1963*c*).

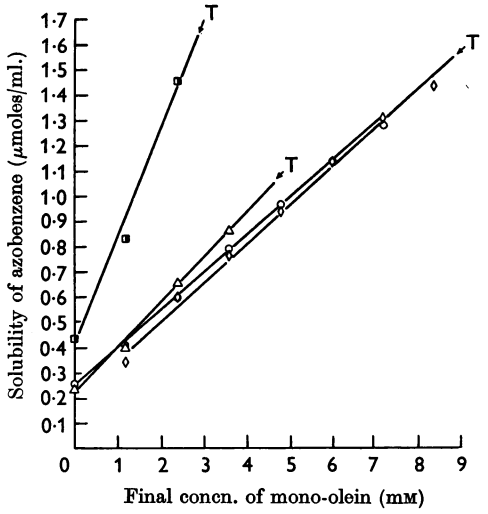

Fig. 4. Linear increase in azobenzene solubility as mono-olein is added to micellar solutions of conjugated bile salts or that of a typical anionic detergent (pH 6·3; concn. of Na^+ ion, 0·15 M; 37°). △, Sodium taurocholate (10 mM); ○, sodium taurodeoxycholate (6·4 mM); ◇, 'simulated intestinal content' (8 mM); ◨, sodium *n*-octylbenzene-*p*-sulphonate (6·4 mM). T indicates the appearance of turbidity, i.e. the solubility of mono-olein in these solutions.

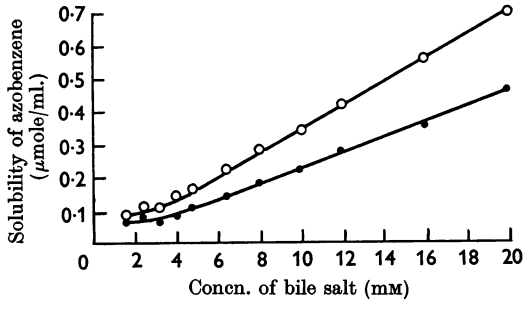

Fig. 5. Solubilization curve of azobenzene in sodium glycochenodeoxycholate at 23° (●) and 37° (○). The Na^+ ion concentration was 0·15 M; the pH was 6·3.

Effect of temperature. Azobenzene has a lower solubility in dilute bile salt solutions at 23° than at 37°. A representative experiment with sodium glycochenodeoxycholate is shown in Fig. 5. The temperature effect at extremely low bile salt concentrations is due to the decrease in the solubility of azobenzene in buffer. Its solubility at 37° is 90 μm-moles/ml., and at 23° about 65 μm-moles/ml. (these values apply only to the experimental conditions described). The curves are divergent, however, indicating a lower saturation ratio at 23° than at 37°. Thus at high bile salt concentration the difference in solubility at these two tempera-

tures will be explained chiefly by the differing saturation ratios. No change in critical micellar concentration was observed.

A temperature coefficient for micellar solubility may be defined by dividing the saturation ratio at 37° by that at 23° (McBain & Hutchinson, 1955), giving: for sodium taurodeoxycholate, 1·61; for sodium glycodeoxycholate, 1·64; for sodium glycochenodeoxycholate, 1·49; for sodium taurodeoxycholate plus increasing amounts of mono-olein, 1·65; and for 'simulated intestinal content', 1·44. The value for 'simulated intestinal content' cannot be determined accurately because of the non-linearity of the solubilization curve.

With mono-olein there was no temperature effect on solubility. The following concentrations of bile salts were studied: sodium taurodeoxycholate, 2·4, 3·2 and 6·0 mM; sodium glycodeoxycholate, 2·4, 3·2, 4·0, 4·8 and 6·0 mM; 'simulated intestinal content', 6·0 and 8·0 mM.

Effect of Na^+ *ion concentration.* (1) Azobenzene. With a fixed bile salt concentration well above its critical micellar concentration an increase in Na^+ ion concentration caused an increase in azobenzene solubility. This effect is greatest at very low Na^+ ion concentrations and of progressively smaller magnitude with higher Na^+ ion concentrations (Fig. 6). In the region of physiological Na^+ ion concentrations (0·13–0·17 M) the effect of Na^+ ion concentration is small. The solubility values shown in Fig. 6 include the solubility of azobenzene in buffer. This falls slightly as the Na^+ ion concentration is increased (Hartley, 1938*b*) but this effect is negligible at the bile salt concentrations used.

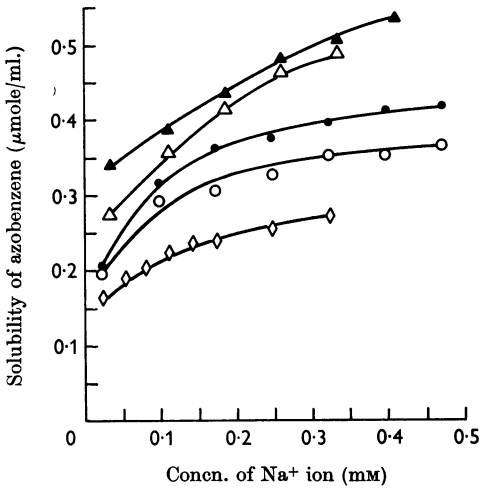

Fig. 6. Effect of total Na^+ ion concentration on the solubility of azobenzene in bile salt solutions (pH 6·3; 37°). ○, Sodium taurodeoxycholate (8 mM); ●, sodium glycodeoxycholate (8 mM); △, sodium taurocholate (20 mM); ▲, sodium glycocholate (20 mM); ◇, 'simulated intestinal content' (8 mM).

A somewhat greater effect of Na^+ ion concentration on azobenzene solubility was noted for the dihydroxy conjugates.

The effect of Na^+ ion concentration on micellar solubility is complex (McBain & Hutchinson, 1955) and has been attributed to the sum of two factors: a lower critical micellar concentration, probably caused by a decrease in thickness of the double layer around the charged groups of the detergent anions (Tartar, 1962), as well as a higher saturation ratio, conveniently explained by 'salting out' of the solute into the pseudophase of the micelle interior (Shinoda & Hutchinson, 1962).

To assess the individual contribution of each of these factors, complete solubilization curves were obtained for three different Na^+ ion concentrations with sodium glycocholate. The shape of the solubilization curve with this bile acid (see Fig. 1) made determination of the critical micellar concentrations difficult, however. For low Na^+ ion concentrations, at which added electrolyte caused a marked increase in azobenzene solubility, the increased micellar solubility was caused by both a lowering of the critical micellar concentration and an increase in the saturation ratio. At higher Na^+ ion concentrations, where added electrolyte influenced azobenzene solubility little, the azobenzene solubility increase was caused by a lowered critical micellar concentration, which shifted the entire solubilization curve to the left without altering its slope.

(2) Mono-olein. The effect of increasing Na^+ ion concentration on mono-olein solubility in bile salt solution was studied by using sodium taurodeoxycholate (2·4 mM) and sodium glycodeoxycholate (6 mM). With both, mono-olein solubility increased slightly and roughly linearly with Na^+ ion concentration. The following values for mono-olein solubility in sodium glycodeoxycholate (6 mM) were obtained: for 21 mM-Na^+ ion, 6·35 μmoles/ml.; for 96 mM-Na^+ ion, 7·25 μmoles/ml.; for 171 mM-Na^+ ion, 8·16 μmoles/ml. At higher Na^+ ion concentrations the rise in turbidity when excess of amphiphile was added was very gradual at first, then increased abruptly.

Effect of added Ca^{2+} *ions.* The addition of Ca^{2+} ions to give concentrations up to 10 mM did not influence the solubility of azobenzene in 5·6 mM-sodium glycodeoxycholate. Similarly, the addition of Ca^{2+} ions to give a concentration of 15 mM had no influence on mono-olein solubility in 2·4 mM-sodium glycodeoxycholate or 2·4 mM-sodium taurodeoxycholate. When, however, Ca^{2+} ions were added to give a concentration of 5 mM in solutions of the typical anionic detergents, sodium *n*-octylbenzene-*p*-sulphonate or sodium oleyl taurate, each

at a concentration of 5·6 mM, the detergent was precipitated out as the calcium salt.

Effect of added glucose. The addition of glucose to give concentrations up to 100 mg./ml. caused a slight fall in the solubility of azobenzene in buffer, but did not influence significantly its micellar solubility.

DISCUSSION

The higher critical micellar concentration associated with the presence of an additional group in the molecule of the cholate conjugates is in agreement with similar observations on paraffin-chain soaps, where the presence of an hydroxyl group is associated with a higher critical micellar concentration (Klevens, 1953). The critical micellar concentrations for the deoxycholate conjugates are slightly but consistently lower than those of the chenodeoxycholate conjugates; the reason for this is unknown. The critical micellar concentrations of the glycine conjugates are the same or slightly lower than those of their corresponding taurine conjugates, but the critical micellar concentrations of detergents with the same hydrophobic moiety but with different charged groups are usually rather similar (Klevens, 1953; McBain & Hutchinson, 1955).

The studies of Ekwall (1951) showed the critical micellar concentration of sodium deoxycholate to be lower than that of sodium cholate, and this observation was subsequently confirmed in the same Laboratory by Sjöblom (1956). The only complete study of the critical micellar concentrations for all of the common conjugated bile salts is that of Norman (1960), who used bile acids of high purity and studied the solubilization of 20-methylcholanthrene. His results are similar to those reported in the present paper, although higher values were obtained because the studies were done without added electrolyte.

Besides having a lower critical micellar concentration, the dihydroxy conjugates also have a higher saturation ratio than the trihydroxy conjugates (Table 1). The higher saturation ratio is not explained by the lower critical micellar concentration, but probably indicates a difference in micellar size or structure or both.

Saturation ratios for the solubilization of cholesterol, which probably behaves as a non-polar solute, have been published by Yoshimuta (1961). Norman's (1960) values for 20-methylcholanthrene solubilization are sufficiently detailed to enable the calculation. Neither of these studies can be compared directly with the present work because of different experimental conditions as well as solutes. However, they are both in general agreement.

Ekwall *et al.* (1957) have studied in detail the solubility of substances such as *n*-decanol in sodium cholate and sodium deoxycholate solution. They have shown that there are sharp changes in the saturation ratio with increasing bile salt concentration, and that at very high bile salt concentrations the saturation ratio of the trihydroxy conjugates equals or exceeds that of the dihydroxy conjugates. The concentrations used in the present study were too low to note such an effect. However, Fig. 1 indicates that the saturation ratios of the trihydroxy conjugates, as well as 'simulated intestinal content', increase with concentration. No change in saturation ratio with concentration was apparent for the dihydroxy conjugates.

Figs. 1–3 and Table 1 show two major differences between solutions of bile salts and those of typical anionic detergents with respect to the solubilization of azobenzene. The bile salts have a lower saturation ratio and their solubilization curves demonstrate a much more gradual inflexion at the critical-micellar-concentration region.

In micelle formation by typical anionic or cationic detergents, intermolecular forces between the amphipathic molecules are considered to play no significant role (Garrett, 1961), the driving force for micelle formation being the increase in entropy resulting from 'melting' of the water structure about the unassociated hydrocarbon chains when the hydrocarbon chain is transferred to the interior of the micelle. Intermolecular forces, such as hydrogen bonds, are probably important for the micellar aggregation of bile salts, and bile salt micelles seem to be analogous to those formed by certain symmetrical dye molecules, where intermolecular forces are of major importance (cf. Hartley, 1936). Experiments have shown that 6 M-urea markedly inhibits micelle formation by conjugated bile salts, but has little influence on typical anionic detergent micelles (A. F. Hofmann, unpublished work). It is convenient to think of the bile salt micelle as being a highly organized aggregate whose effective liquid-hydrocarbon volume is a smaller fraction of the total micellar volume than that of the typical liquid detergent micelle.

When excess of mono-olein is added to a bile salt solution, turbidity occurs because of the occurrence of coacervation. Although, on centrifuging, a viscous layer can be obtained, it is not birefringent. The turbid state maintains its physical properties down to temperatures as low as 15°, far below the melting point of mono-olein. An ordinary emulsion should not exist below the melting point of a substance. The turbid phase of a mono-olein–glycocholate–water system was separated by ultracentrifuging and analysed (kindly performed by Dr L. Mandel and Dr I. Danielsson at the Åbo Akademi, Åbo, Finland). Its composition by weight was: mono-olein, 25 %; glycocholate, 5·2 %; water, 69·5 %. The results are inconsistent with the phase being an emulsion.

There are structural requisites for a substance to exhibit amphiphilic behaviour in bile salt solutions, but clarification of these must await understanding of the molecular arrangement of the bile salt micelle. Oleyl alcohol, 1,2- or 1,3-diolein, or triolein, do not exhibit amphiphilic behaviour in bile salt solutions. 1- and 2-Mono-olein, glycol mono-oleate, lecithin and selachyl alcohol (glycerol 1-mono-oleyl ether) exhibit amphiphilic behaviour. 1-Monolaurin and 1-monoelaidin are amphiphiles in bile salt solutions at 37° but not at 23°, as they then crystallize out (Hofmann, 1963*c*). 1-Monomyristin and 1-mono-palmitin are not amphiphilic at 37° but are at higher temperatures. The minimum temperature at which amphiphilic behaviour is observed is actually the eutectic point of the detergent–amphiphile complex (Lawrence, 1961).

The increase in azobenzene solubility as amphiphile is added to the bile salt micelle is striking, and it is possible that a change in micellar structure occurs as well as a simple increase in size. In agreement with this possibility are the observations of Ekwall & Fontell (1957), who examined the X-ray-diffraction pattern of sodium cholate solutions containing increasing amounts of decanol. When large amounts of decanol were solubilized, there was a fairly sudden change in the position of the intensity maxima.

The increased solubility of a non-polar solute by the addition of amphiphile to the bile salt micelle is a general phenomenon. It has been known for many years that the solubility of cholesterol in bile salt solutions is greatly increased by the addition of lecithin (Spanner & Bauman, 1932; Isaksson, 1953; Johnston & Nakayama, 1957; Yoshimuta, 1961).

This effect can also be observed, but to a smaller extent, with the typical anionic detergents studied, because much less mono-olein can be dissolved in their micelles without the occurrence of turbidity. The property of dissolving a very large quantity of mono-olein is thus another major difference between bile salt micelles and those of ordinary anionic detergents.

No influence of pH on azobenzene or mono-olein solubility was observed, and indeed with a non-ionizable solute no pH effect would be expected.

Bile salts behave as typical anionic detergents with respect to the differences in saturation ratio at 37° and 23°, and the values observed for bile salts agree closely with those reported for comparable temperature differences with non-polar solutes in experiments with anionic detergents (McBain & Hutchinson, 1955). The higher saturation ratio at 37° need not indicate any change in the state of micellar aggregation. The identical solubility of 1-mono-olein at 37° and 23° is consistent with this view.

The micellar solubility of a non-ionizable solute should not be influenced by the addition of Ca^{2+} ions; no effect was observed. The calcium salts of the conjugated bile salts are sufficiently soluble for the micellar state of aggregate to be the preferred form. As noted, the sulphonate and sulphate detergents formed insoluble calcium salts. The resistance of conjugated bile salt solutions to added Ca^{2+} ions may have considerable physiological importance.

The studies of Frazer, Schulman & Stewart (1944) showed that the emulsifying properties of bile salt solutions were poor until amphiphile (in this case saturated monoglyceride and unsaturated fatty acid) was added. Dasher (1952) showed that the surface activity of bile salt solutions was greatly increased by the addition of mono-olein and that a bile salt–mono-olein solution could be compared in this respect with a solution of typical anionic detergent. These experiments show a third parameter in which bile salt solutions containing amphiphile resemble typical anionic detergent solutions, namely their solvent power for non-polar solutes. Thus the bile salt micelle seems to be unique in possessing an extraordinary capacity for dissolving certain polar lipids. Physiologically, this is probably of considerable importance in fat absorption (Hofmann & Borgström, 1962). Its ability to dissolve phospholipids, similarly, may explain the value of bile salt solutions for the disruption of cellular components.

SUMMARY

1. Micellar solubilization by bile salts of fatty acids and monoglycerides has been proposed as an important step in the absorption of dietary fat. The solvent properties of dilute (less than 20 mM) bile salt solutions *in vitro* have been studied for two model solutes: a non-polar solute, *trans*-azobenzene (azobenzene), and a polar or amphiphilic solute, glycerol 1-mono-oleate (mono-olein). Six conjugated bile salts and a mixture simulating the bile salt present in human-intestinal content were compared with three typical anionic detergents.

2. The critical micellar concentration of each bile salt and 'simulated intestinal content' with azobenzene or mono-olein as solute was determined. The saturation ratio (moles of micellar azobenzene or mono-olein/mole of micellar bile salt or detergent) was calculated. For both solutes, dihydroxy conjugates had a lower critical micellar concentration and a higher saturation ratio than the trihydroxy conjugates.

3. The bile salt micelle had a lower saturation ratio for azobenzene but a much higher saturation ratio for mono-olein than the typical anionic detergent micelle. The solvent power of the bile salt

micelle for azobenzene was increased greatly by the addition of mono-olein.

4. There was no effect of pH on the solubility of azobenzene or mono-olein in bile salt solutions.

5. The micellar solubility of azobenzene rose sharply, then levelled with increasing Na^+ ion concentration. The rapid increase was chiefly attributable to an increased saturation ratio. The solubility of mono-olein in bile salt solutions increased slightly with increasing Na^+ ion concentration.

6. Azobenzene was less soluble at 23° than at 37° in bile salt solutions. This decrease was caused by a fall in its solubility in buffer and a lower saturation ratio. The former factor was dominant at low bile salt concentrations, the latter at high bile salt concentrations. Mono-olein had an identical solubility at 23° and 37° in bile salt solutions.

7. Bile salts were not precipitated from solution by the addition of Ca^{2+} ions, under conditions where typical anionic detergents were.

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